PERGAMON

Phytochemistry 57 (2001) 1005–1011

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Acidolysis and hot water extraction provide new insights into the composition of the induced "lignin-like" material from squash fruit

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Received 28 October 1999; received in revised form 6 June 2000

Abstract

Accumulation of "lignin-like" material (L-LM) by plant tissues in response to injury or disease has been observed in a wide variety of plant taxa. The most intensively studied L-LM is that produced by members of the Cucurbitaceae; this material is thought to be an unusual lignin rich in *p*-coumaryl alcohol derived subunits. Employing acidolysis we found the primary degradation product of L-LM from squash fruit was *p*-coumaryl aldehyde. These findings conflict with the current concept of L-LM, but would be consistent with L-LM being a polymer derived directly from *p*-coumaryl aldehyde or a gum containing this compound. Results of hot water extraction support the latter possibility. Further, we report on a simple TLC method useful for rapid qualitative characterization of acidolysis degradation products.

Keywords: *Cucurbita maxima*; Cucurbitaceae; Squash; Composition of induced lignin; *p*-Coumaryl alcohol; *p*-Coumaryl aldehyde

1. Introduction

The lignin-like material (L-LM) produced by many plants in response to injury or disease has been studied by numerous investigators and is frequently associated with resistance to fungal or bacterial pathogens (Ride, 1975; Vance et al., 1980; Nicholson and Hammerschmidt, 1992). Lignins are a complex class of compounds derived via the free radical polymerization of the three primary lignols (Fig. 1), *p*-coumaryl **1b**, coniferyl **2b** and sinapyl **3b** alcohols (Harkin, 1967; Freudenberg and Neish, 1968; Whetten et al., 1998). Lignins are classified based on the ratio of the different lignols present. Because of their high molecular weight and insolubility, precise knowledge of the composition of lignins cannot be obtained directly, but must be gained indirectly by NMR of soluble lignin fractions or by identification of lignin degradation products (Lewis and Yamamoto, 1990). Degradation methods used include pyrolysis-GC–MS, hydrolysis, and oxidation (Tanahashi and Higuchi, 1988; Ralph and Hatfield, 1991). In addition to providing subunit composition, a portion of the products from the first two methods retain their C-3 side chains, providing information about the interunit linkages in the parent lignin (Lundquist, 1992; Ralph and Hatfield, 1991). However, the sheer number of possible products complicates routine analysis of lignins by these methods. In contrast, oxidative methods degrade lignins to the benzaldehyde or benzoic acid analogues of their parent lignols. Because there are only three primary products, analyses of oxidative degradation products are relatively simple, (Tanahashi and Higuchi, 1988), but information on the nature of the sidechains is lost.

Deposition of L-LM has been reported in a wide variety of plant taxa (Vance et al., 1980; Rittinger et al., 1987). However, only in a relatively small number of plants has the composition of the L-LM been examined. The L-LM from members of the Cucurbitaceae is perhaps the best characterized, having been studied by four independent research groups using alkaline cupric oxide or nitrobenzene oxidization methods. L-LM induced by fungal infection in five species of cucurbits was

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0031-9422/01/$ - see front matter
PII: S0031-9422(01)00096-6

characterized by Hammerschmidt et al. (1985) and in muskmelon by Grand and Rossignol (1982). L-LM in cucumber, induced by wounding of fruit (Walter et al., 1990) or by an elicitation of hypocotyls with a pectic elicitor (Robertsen and Svalheim, 1990) has also been characterized. In all these studies, degradation products of tissues containing L-LM contained an abundance of *p*-hydroxybenzaldehyde, with increases of up to 61-fold over control tissues (Grand and Rossignol, 1982; Hammerschmidt et al., 1985; Robertsen and Svalheim, 1990; Walter et al., 1990). Similar results have been reported in other plant taxa. Compared to controls, degradation products of L-LM-containing tissues of radish roots, wheat leaves, soybean cotyledons, and parsley suspension cells had 2.5, 6.7, 2.9, or 8.6 fold more *p*-hydroxybenzaldehyde (Asada and Matsumoto, 1972; Ride, 1975; Graham and Graham, 1991; Kauss et al., 1993). Thus, our current concept about L-LM is of a lignin that is unusually rich in *p*-coumaryl alcohol derived subunits. Consistent with their role in defense, lignins rich in this lignol may yield highly condensed lignins that are resistant to degradation (Hammerschmidt et al., 1985). Some lignins of unique function, such as compression wood lignin, also have unique compositions (Nimz et al., 1981; Timell, 1982, 1986).

Although there is a consensus that the function of L-LM is protective, investigators earlier in this century had a much different concept of its chemical nature. While these earlier investigators did not have a precise chemical model, the intense reaction with phloroglucinol-HCl (PG-HCl) was attributed to the presence of aromatic aldehydes held within a water insoluble gum, termed wound gum (Bloch, 1937; Rawlins and Takahashi, 1952). Both wound gum and lignin become strikingly colored in PG-HCl; however, on the basis of other histological, anatomical and developmental criteria, wound gum was thought to be biochemically distinct from lignin (Hewitt, 1938; Schneider, 1980).

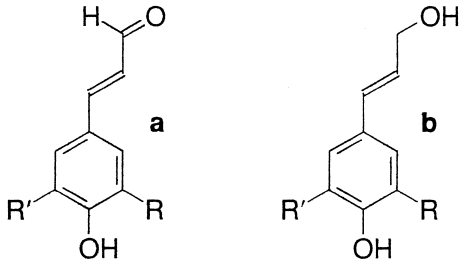
We recently reported that squash tissue elicited with pectinase synthesizes and accumulates the antifungal compound *p*-coumaryl aldehyde **1a** (Stange et al., 1999). The appearance of this compound is coincidental with the deposition of L-LM (unpublished data). This compound is also the immediate biosynthetic precursor of *p*-coumaryl alcohol that is presumed to be the primary component of L-LM in cucurbits (Lewis and Yamamoto, 1990). Recent studies of novel lignins, formed by plants with reduced cinnamyl alcohol dehydrogenase activity, have dramatically expanded our concept of lignins (Whetten et al., 1998). In these plants, conversion of coniferyl aldehyde **2b** to coniferyl alcohol **2a** is partly or completely blocked and their lignins are synthesized, in part, from coniferyl aldehyde (Higuchi et al., 1994; Baucher et al., 1996; Ralph et al., 1997). Thus, aldehyde analogs of lignols can be directly polymerized into lignins or lignin-like materials. The oxidation methods used by earlier investigators to characterize the L-LM from cucurbits would not have differentiated between lignins derived from *p*-coumaryl aldehyde or alcohol (Grand and Rossignol, 1982; Hammerschmidt et al., 1985; Robertsen and Svalheim, 1990; Walter et al., 1990). Distinguishing the identity of L-LM's parent compounds requires use of methods which do not destroy the C3 side chain.

The close association between *p*-coumaryl aldehyde and L-LM accumulation in squash, together with the general doubts of earlier researchers that L-LM is even lignin at all, poses several fundamental questions. Is the L-LM an unusual lignin derived from *p*-coumaryl alcohol or aldehyde? Or, in fact, is the material a lignin at all? We attempt to begin to address these questions by using acidolysis to analyze tissues containing L-LM. In addition, we report on a TLC method for separation of the acidolysis degradation products which greatly simplifies the analyses of the hydrolysates of L-LM.

2. Results and discussion

Identification of components unique to L-LM depends on comparing degradation products of tissues having low lignin content with or without L-LM being present. Further, comparison of degradation products from L-LM containing tissue with highly lignified tissues would assist in the characterization of L-LM acidolysis products and provide confirmation of the methodology. Our experimental scheme utilized extractive-free cell wall preparations from squash fruit tissue elicited with pectinase and cured 24 h to promote accumulation of L-LM (Stange and McDonald, 1999), from unelicited freshly sliced fruit tissue, and from tissue of the highly lignified stem of the fruit.

Histochemical tests were performed to confirm the composition of these cell wall preparations. When the Mäule test was performed, the abundant, heavily sclerified cell walls in the stem tissue gave an intense reaction,



		R	R′
1a/b	*p*-coumaryl aldehyde/alcohol	H	H
2a/b	coniferyl aldehyde/alcohol	OMe	H
3a/b	sinapyl aldehyde/alcohol	OMe	OMe

Fig. 1. Primary lignols and their corresponding aldehydes.

turning a dark purple-red (Fig. 2, Mäule). However, microscopic examination revealed that remnants of xylem elements in the stem tissue gave no reaction with the Mäule test. Similarly, positively testing xylem components were observed in neither fresh nor elicited-cured fruit tissue preparations. The Mäule (-) reaction of the xylem is consistent with the observations of Walter et al. (1990) and with the results of Robertsen and Svalheim (1990), who reported finding no syringaldehyde in the CuO oxidation products of cucumber hypocotyls. Syringaldehyde is indicative of sinapyl alcohol **3b** derived constituents in lignin, which in turn, form colored products in the Mäule test (Iiyama and Plant, 1988). However, the xylem content of the stem tissue was low, relative to the abundant sclerified cells, which composed most of the sample.

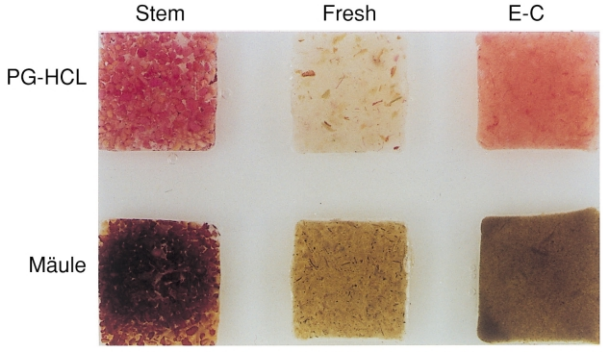


Fig. 2. Histochemical reactions of cell wall preparations. Each well contains similar volumes of extractive-free cell wall preparations from stem (Stem), fresh fruit (Fresh) and elicited-cured (E–C) fruit tissues. Cell wall preparations were subjected to the phloroglucinol-HCl test (PG-HCl) for aldehydes (upper) and the Mäule test for syringyl groups (lower). Wells are 1 cm^2.

Both sclerified cells and xylem remnants in the stem tissue sample reacted positively with PG-HCl, turning a purple-red color (Fig. 2, PG-HCl). The sclerifed tissue in the stem sample reacted positively with both reagents; this set of reactions is typical of angiosperm lignins (Towers and Gibbs, 1953; Lewis and Yamamoto, 1990). In fresh fruit cell wall preparations, only xylem elements reacted positively with PG-HCl. In contrast, a large portion of the elicited-cured fruit tissue reacted with the PG-HCl reagent, L-LM turned red while the xylem elements turned a purple-red color. The abundance of xylem in both fresh and elicited-cured fruit samples was quite low (Fig. 2, PG-HCl). The PG-HCl (+) and Mäule (-) histochemical reactions of L-LM are consistent with those reported by previous workers in cucurbits (Walter et al., 1990) and other crops (Schneider, 1980). Thus, the fruit tissue preparations provide samples with a low abundance of vascular lignin, with L-LM being either abundant in elicited cured tissue, or absent in fresh tissue. The stem sample has no L-LM, but contains an abundance of typical lignin.

Acid hydrolysis products of these three types of samples were obtained by the standard method, refluxing in dioxane-HCl (Tanahshi and Higuchi, 1988; Lundquist, 1992) and by mild acid hydrolysis [2 N HCl at 90°C]. Degradation products from both methods were separated by TLC and visualized with PG-HCl or Folin–Ciocalteu's phenol reagent. PG-HCl gives red to purple products with aromatic aldehydes and yellow to orange spots with furfurals (Feigl and Anger, 1966). Several compounds giving red to purple hued spots were observed in the stem tissue sample (Fig. 3). Two of these spots corresponded in mobility and color to the magenta spot of coniferyl aldehyde **2a** and the purple-brown spot of sinapyl aldehyde **3a**. Large quantities of staining material were

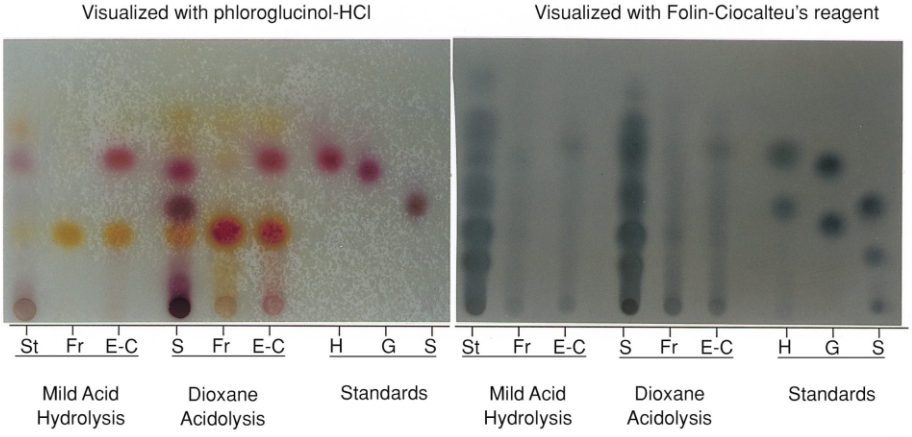


Fig. 3. Comparison of mild acid hydrolysis and dioxane acidolysis products from stem (St), fresh fruit (Fr) and elicited-cured fruit (E-C) extractive-free cell wall preparations. Standards are mixtures of *p*-coumaryl (H) **1**, coniferyl (G) **2** or sinapyl (S) **3** alcohol and aldehyde; 0.5 μg of each compound spotted. Plates were developed in hexane–EtOAc (1:1); solvent fronts travelled 90 mm, 60 mm of travel shown. TLC plate on left visualized with phloroglucinol-HCl to reveal aromatic aldehydes (red to purple colors) and furfurals (orange to yellow colors). Loading of hydrolysates equivalent to 1.5 mg cell wall preparation. TLC plate on right visualized with Folin–Ciocalteu's reagent to reveal phenolics. Loadings of hydrolysates equivalent to 0.5 mg cell wall preparation.

present at the origin, indicating that component(s) of the sample were of higher molecular weight or highly polar. No red hued spots were evident from the fresh fruit samples; this would be expected given the low abundance of lignin in the sample. The elicited-cured tissue yielded a single red spot, unique to this sample. This compound had similar color and mobility to *p*-coumaryl aldehyde **1a**. When plates were visualized for phenolics with Folin-Ciocalteu's reagent, similar patterns were observed. Products of the stem tissue sample were abundant and numerous (Fig. 3). By contrast, in fresh and elicited-cured fruit tissue samples products were far less abundant, indicating that very few phenolic components were present. The exception to this was *p*-coumaryl aldehyde **1a** in the elicited-cure sample, which produced a distinct spot with Folin–Ciocalteu's reagent. Both acid hydrolysis methods yielded similar products from fresh and elicited-cured fruit samples, but for the stem sample mild acid hydrolysis yielded fewer products and gave lower yields than dioxane acidolysis.

Hot water and mild acid hydrolysis of elicited tissue yielded 161 ± 25 ($n = 4$) and 303 ± 19 ($n = 4$) μg *p*-coumaryl aldehyde **1a** per gram cell wall material, respectively. When residues from hot water hydrolysis were subjected to mild acid hydrolysis, additional *p*-coumaryl aldehyde (130 ± 25 μg/g cell wall) was recovered. Because the hot water suspension was slightly acidic (initial pH, 5.7; final pH, 5.2), one possibility is that the *p*-coumaryl aldehyde recovered by this treatment was held by relatively easily hydrolyzed covalent bonds, such as ester linkages. However, the more probable interpretation is that the compound was contained within a gum which was dissolved by the hot water. We are attempting to address this technically difficult question presently.

A single furfural predominated in both fruit samples (orange spots, Fig. 3, PG-HCl), but furfurals were rare or lacking in the stem tissue sample. The abundance of furfurals in fruit samples was much greater with the dioxane acidolysis method than with mild acid hydrolysis.

GC–MS analysis of the stem tissue sample acidolysis products identified six guaiacyl and two syringyl derivatives. The presence of syringyl units is consistent with the positive Mäule reaction of the tissue (Iiyama and Plant, 1988). In the fresh fruit sample, no lignin related degradation products were found by GC–MS, again indicating the low abundance of lignified xylem in the fresh fruit samples. The identity of the compound giving a red colored spot in TLCs of the elicited-cured samples was confirmed by GC–MS and by NMR following separation by TLC. As suspected, the compound was *p*-coumaryl aldehyde **1a**. Further, this was the only lignin related product detectable by GC–MS.

The predominant furfurfal in both the fruit samples was 5-(hydroxymethyl)-2-furaldehyde. In addition, the elicited-cured sample contained 2-hydroxymethyl furan. Presence of this compound in the mixture interfered with the identification of *p*-coumaryl aldehyde **1a** by GC–MS. We are uncertain of the significance of this compound, but it may indicate differences in the carbohydrate composition between fresh and elicited-cured samples.

Our results confirm that the stem tissue contained a typical angiosperm lignin, composed primarily of guaiacyl and syringyl units. The fresh fruit tissue had a very low abundance of lignin. These results could be anticipated based on histochemical tests and microscopic observations. The colors developed by hydrolysates when TLC plates were visualized with PG-HCl corresponded very well to the colors of cell wall residues treated with this reagent. The elicited-cured tissue developed the same pure red color developed by *p*-coumaryl aldehyde **1a**. The stem tissue's more purple color suggests the magenta to purple colors developed by coniferyl **2a** and sinapyl **3a** aldehydes.

Finding *p*-coumaryl aldehyde as the sole acidolysis product of L-LM was unexpected. This result is strong evidence that L-LM is not derived from *p*-coumaryl alcohol **1b**. The exact nature of L-LM is, however, much less clear. If it were a lignin-like material derived from *p*-coumaryl aldehyde, we would have expected more related products, similar to the numerous products yielded from stem tissue. Because a *p*-coumaryl aldehyde based lignin might be highly condensed, these products could be quite rare, and more exhaustive are studies needed to determine if they exist. Attempts to dissolve the L-LM in acetyl bromide and subsequent application of the 'derivatization followed by reductive cleavage' method (Lu and Ralph, 1997a,b,1998) were unsuccessful, even with extended treatment periods. Only minor amounts of the fraction, mainly saccharide components, dissolved. We (Lu and Ralph, 1999, unpublished) have previously encountered difficulty in dissolving aldehyde-rich fractions in acetyl bromide. While our results do fit well with the concept of the material being a water-insoluble gum imbedded with *p*-coumaryl aldehyde, they do not rule out the possibility that a portion of this compound is covalently bound. Whatever it is, this material certainly differs from a lignin derived from *p*-coumaryl alcohol **1b**. More fundamental research, utilizing a wider variety of approaches based on the above models, is needed to determine the actual composition and structure of the so-called "lignin-like" material from elicited cured squash.

3. Experimental

3.1. Plant material, elicitation and isolation of lignin-like material

Green acorn squash, *Cucurbita maxima*, were purchased from retail outlets. Only recently harvested fruits

with green living stems were selected. Stems were air dried and ground through a #20 mesh screen using a Wylie® mill. Fresh fruit tissue was diced finely. Elicited-cured tissue was prepared as described earlier from squash sliced 5 mm thick (Stange et al., 1999). Extractive-free cell wall preparations were made by first homogenizing tissues 1 min in 10 mM phosphate buffer, pH 6 (25 ml per g dry wt). Cell wall material was pelleted by centrifugation (6000×*g*) and washed with two changes of buffer. The water-insoluble residue was further extracted with two changes over 24+ h of the following sequence of solvents: ethanol–water (4:1) (25 ml per g dry wt); chloroform–methanol–water (5:10:4); methanol; acetone (12 ml per g dry wt). Extractive-free cell wall preparations were stored at −20°C.

3.2. Chemicals

Coniferyl and sinapyl alcohols and aldehydes were purchased from commercial sources. *p*-Coumaryl alcohol **1b** was synthesized according to Quideau and Ralph (1992) and *p*-coumaryl aldehyde was prepared from **1b** by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

3.3. Histochemical tests

PG-HCl and Mäule tests were performed on cell wall preparations using standard methods (Rawlins and Takahashi, 1952). Microscopic examination of samples was done under bright and dark field illumination using a Nikon Optiphot microscope at 40 and 100× magnification.

3.4. Hydrolysis of extractive-free cell wall samples

A modification of the procedure of Tanahashi and Higuchi (1988) was used for acidolysis (Lundquist, 1992). Tissue, 400 mg, was refluxed under N_2 in 20 ml dioxane–2 M HCl (9:1) for 3 h. The suspension was filtered, and the residue washed with dioxane. The filtrate was adjusted to pH 3 with 0.4 M $NaHCO_3$ and stirred for 30 min. This solution was extracted with methylene chloride, and the organic phase was dried over Na_2SO_4, filtered, and evaporated under vacuum. Acidolysis products were dissolved in EtOAc–EtOH (3:1) and stored at −20°C.

For mild acid hydrolysis, 400 mg extractive-free cell wall residue was placed in a 100-ml volumetric flask with 40 ml 2 N HCl. The flask was purged with N_2, capped with a marble, and placed in a 90°C water bath for 3 h. The suspension was cooled, 40 ml 2-propanol added, and then filtered. The residue was washed with 20 ml 2-propanol. Combined filtrates were reduced to aqueous under vacuum, extracted 2× with methylene chloride, and the organic phase was dried over Na_2SO_4. This solution was filtered, dried, and the products dissolved and stored as the acidolysis samples above. Hot water hydrolysis products of elicited-cured tissue were also obtained using this procedure by substituting distilled water for 2 M HCl.

3.5. Analyses of hydrolysis products by TLC

Samples and standards were spotted onto polyester backed silica gel TLC plates (250 μm) and developed in hexane–EtOAc (1:1). Phenolics were visualized using Folin Ciocalteu's reagent as described by Fry (1988). Aromatic aldehydes and furfurals were visualized by first spraying with 1% phloroglucinol in ethanol–water (7:3), air drying, and spraying with conc HCl (Feigl and Anger, 1966). The quantity of *p*-coumaryl aldehyde present in mild acid and hot water hydrolysates was determined as follows. Samples and standards of *p*-coumaryl aldehyde were separated by TLC, spots corresponding to the compound were located under long-wave UV, scraped off, and dissolved in 1 ml 95% ethanol. Abundance of *p*-coumaryl aldehyde was quantified by A_{325}.

3.6. Analyses of hydrolysates by GC–MS

GC was run on a Hewlett-Packard (Atlanta, GA) 5980 gas chromatograph; column 0.2 μm film, 0.2 mm × 30 m SPB-5 (Supelco); He carrier gas, 1 ml/min; 30:1 split ratio; injector 220°C, initial column temperature 150°C, ramped at 10°C/min to 310°C, hold for 10 min, total running time 34 min. Mass spectra (EI, 70 eV) were collected on a Hewlett-Packard 5970 MS Detector connected directly to a similar column in the same GC.

Three samples were chosen for GC–MS analysis: mild acid hydrolysate of stem tissue, and dioxane acidolysis hydrolysates of fresh and elicited-cured fruit tissues. For trimethylsilylation hydrolysates (0.5 mg) were silylated using BSTFA {N,*O*-bis(trimethylsilyltrifluoroacetamide)} (80 μl) plus pyridine (20 μl) at 50°C for 15 min. For acetylation, hydrolysates (1 mg) were acetylated with 1:1 acetic anhydride:pyridine (0.5 ml) at RT for 4 h. Solvents were removed by co-evaporation with ethanol on a rotary evaporator. The acetylated products were dissolved in methylene chloride (0.2 ml) for GC.

3.6.1. GC–MS

Acetylated or trimethylsilylated samples (2 μl) were injected into the GC. Trimethylsilylated *p*-coumaraldehyde eluted at 6.01 min; acetylated *p*-coumaryl aldehyde at 5.76 min. Mass spectra were identical with those derived from authentic *p*-coumaryl aldehyde (Aldrich): trimethylsilyl *p*-coumaryl aldehyde 220 (M+, 66), 205 (47), 73 (100); 4-acetoxycinnamaldehyde 190 (M+, 14), 148 (100).

3.7. Confirmation of p-coumaryl aldehyde by NMR

The dioxane acidolysis hydrolysate of elicited fruit tissue (37 mg) was applied to a preparative TLC plate (2 mm thickness, Macherey-Nagel Products, Germany), and eluted with 10:2 CH_2Cl_2:EtOAc. The band at R_f 0.6 was isolated (2.5 mg) and shown by NMR (^{1}H, COSY) to be identical to authentic *p*-coumaryl aldehyde **1a** — NMR data were the same as for compound number 152 in the "NMR Database of Lignin and Cell Wall Model Compounds" (Ralph et al., 1998). The acetylated TLC fraction had spectra identical to acetylated *p*-coumaryl aldehyde, compound number 223 in the same database.

Acknowledgements

We thank anonymous reviewers, D. Akin, N.G. Lewis, W. Walter and C. West for their helpful suggestions on improving this manuscript and Hoon Kim for preparation of the *p*-coumaryl aldehyde. Henry Schneider's efforts in educating the first author on wound gum are greatly appreciated.

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